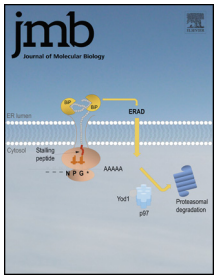

Molecular Mechanisms of Glutaredoxin Enzymes: Versatile Hubs for Thiol–Disulfide Exchange between Protein Thiols and Glutathione

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Abstract

The tripeptide glutathione (GSH) and its oxidized form glutathione disulfide (GSSG) constitute a key redox couple in cells. In particular, they partner protein thiols in reversible thiol–disulfide exchange reactions that act as switches in cell signaling and redox homeostasis. Disruption of these processes may impair cellular redox signal transduction and induce redox misbalances that are linked directly to aging processes and to a range of pathological conditions including cancer, cardiovascular diseases and neurological disorders. Glutaredoxins are a class of GSH-dependent oxidoreductase enzymes that specifically catalyze reversible thiol–disulfide exchange reactions between protein thiols and the abundant thiol pool GSSG/GSH. They protect protein thiols from irreversible oxidation, regulate their activities under a variety of cellular conditions and are key players in cell signaling and redox homeostasis. On the other hand, they may also function as metal-binding proteins with a possible role in the cellular homeostasis and metabolism of essential metals copper and iron. However, the molecular basis and underlying mechanisms of glutaredoxin action remain elusive in many situations. This review focuses specifically on these aspects in the context of recent developments that illuminate some of these uncertainties.

Introduction

Aerobic life is sustained by a complex network of redox processes that use dioxygen as terminal electron acceptor. Consequently, production of reactive oxygen species (ROS) including superoxide radicals (O_2•), hydrogen peroxide (H_2O_2) and hydroxyl radicals (HO•) is inevitable for aerobic organisms. Such species have been linked traditionally to cell damage, but there is now an appreciation of some beneficial effects [1]. Certain ROS have evolved to play important roles in the cell signaling that regulates crucial developmental and physiological events [2,3]. However, their unregulated production may induce oxidative stress and irreparable damage. Indeed, accumulation of this damage is a primary cause of normal aging [4,5] and is central to myriad human diseases including cancers, diabetes and cardiovascular, inflammatory, pulmonary and neurological disorders [6]. Consequently, aerobic organisms have evolved dedicated cellular components and sophisticated regulatory mechanisms to control and maintain an appropriate redox balance [1].

One of the most important cellular components in this context is glutathione (GSH; γ-glutamyl-cysteinyl-glycine) that has evolved to control the redox status of protein thiols [7,8]. Many cell signaling processes involve Cys modification, and oxidative damage to these protein thiols can compromise such processes [9,10].

GSH is abundant in the cytosol (1–10 mM) [11] and is oxidized reversibly to its disulfide form GSSG. The GSSG/2GSH pair forms an important cellular

redox couple. The distribution of GSSG and GSH is dynamic in cells and the ratio (plus the individual concentrations) varies considerably across different subcellular compartments and, consequently, so do the formal reduction potentials that drive diverse redox processes in different directions [12–14]. Cytosolic GSSG is reduced by glutathione reductase at the expense of NADPH and/or is removed efficiently by multiple transport pathways to extracytosolic compartments or vacuoles [13,14]. Consequently, the cytosol may contain very little GSSG and so the formal reduction potentials may be highly negative (below −300 mV) and fluctuate sensitively with GSSG concentrations [14]. On the other hand, the GSSG/GSH ratios in other more oxidizing compartments (such as the endoplasmic reticulum in the secretary pathway) may be much higher (up to ~1:1) with less negative but buffered reduction potentials (~−210 mV) [14,15].

GSSG/GSH may also be exported into extracellular milieux, but the concentrations are much lower, in the μM range [16]. The reduction potential defined by the GSSG/2GSH couple depends not only on the GSSG/GSH ratio but also on the absolute GSH concentration (see Eqs. (1b) and (1b') below). Consequently, although the relative GSH concentration in plasma remains much higher than that of GSSG, the reduction potential of GSSG/2GSH in plasma is much more positive at ~−140 mV [17].

The oxidation status of protein thiols is expected to change dynamically across different cellular compartments as well to meet the requirements of diverse functions and protection of protein thiols. This may be achieved conveniently by reversible *S*-glutathionylation of protein thiols P(SH) to yield mixed disulfides P(SSG) or protein disulfides P(SS) (Fig. 1). *S*-glutathionylation constitutes an important mechanism of antioxidant defense and redox functioning [18]. However, these GSH-mediated protein thiol modifications, albeit reversible, are usually too slow to be biologically viable and require catalysis to ensure rapid cellular responses [19].

Glutaredoxins (Grxs) have evolved as a class of GSH-dependent thiol–disulfide oxidoreductases that facilitate direct reversible redox chemistry between protein thiols and the cellular thiol pool GSSG/GSH. They belong to the thioredoxin superfamily that includes thioredoxins (Trxs), protein disulfide-isomerases (PDIs) and the disulfide bond protein A (DsbA) [20]. They all share a thioredoxin fold with a Cys-xx-Cys active site motif [21]. Crucially, the environment of the solvent-exposed N-terminal Cys in these enzymes induces a low pK_a that allows its thiolate anion to act as a nucleophile to initiate the thiol–disulfide exchange. However, Grxs are unique in that their activity employs GSSG/GSH as a direct electron acceptor/donor [8,20,22]. This is attributed to the existence of at least one distinct GSH interaction site that enables them to act as scaffold proteins for the binding and delivery of GSH [23]. Consequently, Grxs are emerging as a class of versatile enzymes in cell signaling and redox homeostasis and are the subject of many recent reviews [8,20,22,24–29].

Studies of the catalytic functions of Grxs have focused mainly on deglutathionylation (i.e., reduction of P(SSG) functions) [8,20,24,26,28]. However, they also catalyze other types of protein thiol redox processes, such as those summarized in Fig. 1 [14,30–33]. For example, they promote a number of protein disulfide bond reductions and the Trx dithiol mechanism has been assumed to apply to these Grx enzymes [8,20,24–29]. However, it has been demonstrated that, in most cases examined, the N-terminal Cys alone in the active site motif Cys-xx-Cys is sufficient for catalysis with no clear requirement for the C-terminal Cys [31–36]. This raises the following questions: what is the reaction mechanism for protein disulfide reduction and what are the functional roles of the C-terminal Cys in the active site?

On the other hand, certain Grxs are known to play a role in cellular iron homeostasis via formation of ternary complexes involving iron and GSH [37]. The

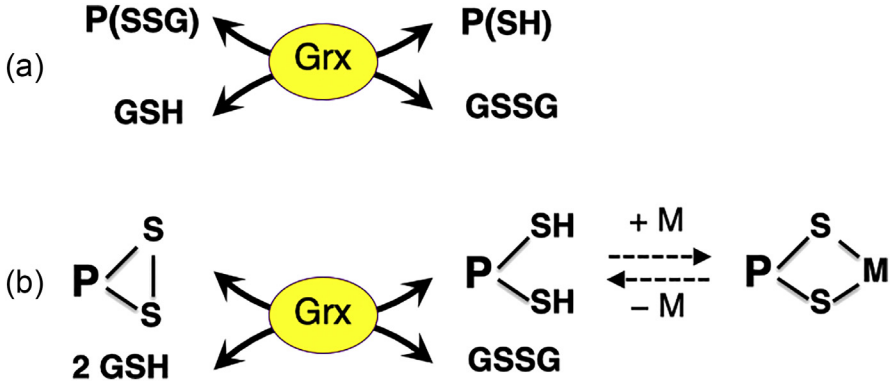


Fig. 1. Thiol–disulfide exchange catalyzed by Grx enzyme between GSSG/GSH and protein thiols in response to oxidative stress for: (a) protein monothiol P(SH) and (b) the protein dithiol $P(SH)_2$ of a metal-binding site.

thioredoxin enzyme family including Grxs has also been demonstrated to bind Cu(I) with femtomolar affinity and may have a role in copper homeostasis and in protection of cells from copper toxicity [31,38–40]. An intact dithiol active site is required for high affinity Cu(I) binding, but this inhibits the enzyme redox activity [31,41,42]. In addition, the C-terminal Cys in the active site is beneficial or essential for catalysis of certain important cellular redox processes [43–45].

Consequently, Grxs must have evolved to possess the flexibility of employing different mechanisms for their various functions under different cellular conditions. This review aims to address these aspects with focus on recent developments. But first, we start with a general discussion of both the thermodynamic and kinetic aspects of thiol–disulfide exchange reactions that are essential for an understanding of the catalytic processes.

General Aspects on Thiol–Disulfide Exchange

Thermodynamic considerations

The net direction of the redox processes in Fig. 1 must be based on favorable thermodynamic gradients: enzymes can overcome an unfavorable kinetic barrier but not a thermodynamic one. Eq. (1) is the classic reaction catalyzed by Grxs and can be factorized into two half reactions 1a and 1b. Consequently, the reaction potential $\Delta E'_{(1)}$ is described by Eq. (1') and derived from Eqs. (1a') and (1b'):

$$\mathrm{P(SSG)} + \mathrm{GSH} \rightleftharpoons \mathrm{P(SH)} + \mathrm{GSSG} \qquad (1)$$

$$\Delta E'_{(1)} = \left(E^{o\prime}_{\mathrm{P(SSG)}} - E^{o\prime}_{\mathrm{GSSG}}\right) - \frac{RT}{2F} \ln\left(\frac{[\mathrm{P(SH)}]}{[\mathrm{P(SSG)}]} \frac{[\mathrm{GSSG}]}{[\mathrm{GSH}]}\right) \qquad (1')$$

$$\mathrm{P(SSG)} + 2\mathrm{H^+} + 2\mathrm{e^-} \rightleftharpoons \mathrm{P(SH)} + \mathrm{GSH} \qquad (1a)$$

$$E'_{\mathrm{P(SSG)}} = E^{o\prime}_{\mathrm{P(SSG)}} - \frac{RT}{2F} \ln \frac{[\mathrm{P(SH)}][\mathrm{GSH}]}{[\mathrm{P(SSG)}]} \qquad (1a')$$

$$\mathrm{GSSG} + 2\,\mathrm{H^+} + 2\,\mathrm{e^-} \rightleftharpoons 2\,\mathrm{GSH} \qquad (1b)$$

$$E'_{\mathrm{GSSG}} = E^{o\prime}_{\mathrm{GSSG}} - \frac{RT}{2F} \ln \frac{[\mathrm{GSH}]^2}{[\mathrm{GSSG}]} \qquad (1b')$$

The equivalent reaction involving an internal disulfide bond P(SS) is Eq. (2) whose reaction potential $\Delta E'_{(2)}$ is described by Eq. (2') and derived from Eqs. (2a') and (1b'):

$$\mathrm{P(SS)} + 2\,\mathrm{GSH} \rightleftharpoons \mathrm{P(SH)_2} + \mathrm{GSSG} \qquad (2)$$

$$\Delta E'_{(2)} = \left(E^{o\prime}_{\mathrm{P(SS)}} - E^{o\prime}_{\mathrm{GSSG}}\right) - \frac{RT}{2F} \ln\left(\frac{[\mathrm{P(SH)_2}]}{[\mathrm{P(SS)}]} \frac{[\mathrm{GSSG}]}{[\mathrm{GSH}]^2}\right) \qquad (2')$$

$$\mathrm{P(SS)} + 2\mathrm{H^+} + 2\mathrm{e^-} \rightleftharpoons \mathrm{P(SH)_2} \qquad (2a)$$

$$E'_{\mathrm{P(SS)}} = E^{o\prime}_{\mathrm{P(SS)}} - \frac{RT}{2F} \ln \frac{[\mathrm{P(SH)_2}]}{[\mathrm{P(SS)}]} \qquad (2a')$$

On the other hand, two different dithiol proteins P1 and P2 may exchange their disulfide bonds directly (see Eqs. (3) and (3')):

$$\mathrm{P1(SS)} + \mathrm{P2(SH)_2} \rightleftharpoons \mathrm{P1(SH)_2} + \mathrm{P2(SS)} \qquad (3)$$

$$\Delta E'_{(3)} = \left(E^{o\prime}_{\mathrm{P1(SS)}} - E^{o\prime}_{\mathrm{P2(SS)}}\right) - \frac{RT}{2F} \ln\left(\frac{[\mathrm{P1(SH)_2}]}{[\mathrm{P1(SS)}]} \frac{[\mathrm{P2(SS)}]}{[\mathrm{P2(SH)_2}]}\right) \qquad (3')$$

$E^{o\prime}_{\mathrm{P(SSG)}}$, $E^{o\prime}_{\mathrm{GSSG}}$ and $E^{o\prime}_{\mathrm{P(SS)}}$ are the standard reduction potentials for the redox couples P(SSG)/(P(SH) + GSH), GSSG/2GSH and P(SS)/$\mathrm{P(SH)_2}$ represented by the half reactions 1a, 1b and 2a, respectively. The directions of Eqs. (1) and (2) are determined primarily by these potentials but also involve the contributions of the molar ratio GSSG/GSH and of the free GSH concentration.

From a molecular viewpoint, the reduction potential of a disulfide/thiol couple is determined primarily by the conformation of the disulfide bond in the oxidized form and by the pK_a values of the thiol groups (-SH) in the reduced form [46,47]. A strained disulfide will release conformational energy upon reduction and so has a more positive potential [47]. Likewise, a lower pK_a for a thiol group indicates a stronger electron-withdrawing effect on the sulfur atom and this also induces a more positive potential [46]. The dipeptide sequence between the two active site Cys residues in a dithiol enzyme plays an important role in modulating both the stability of the disulfide bond and the pK_a of the reactive thiol and, consequently, the reduction potential [48].

A consensus value of $E^{o\prime}_{\mathrm{GSSG}}$ (−240 mV at pH 7.0) for the redox couple GSSG/2GSH has been reached and been used frequently as a calibration standard for determination of reduction potentials of other thiol redox couples [49]. The $E^{o\prime}_{\mathrm{P(SS)}}$ values for the half reaction of many dithiol enzymes and proteins (see

Eqs. (2a) and (2a')) have been determined based on thiol–disulfide exchange equilibria 2 and/or 3 (Table 1). However, it can be a difficult challenge to quantify these equilibria reliably if the reactions need to be fixed ("quenched") via a chemical reaction for subsequent speciation analysis [32,57]. The reasons include the following: (i) the quenching reaction may not be able to stop the exchange reaction quickly enough without inducing some redox shifting, especially for those fast thiol–disulfide exchange reactions involving a redox enzyme; (ii) some reactions may be too slow to reach equilibrium, and therefore, it is important to conduct the same exchange reaction in opposite directions to ensure consistency; and (iii) the speciation analysis employed may lack adequate accuracy. In addition, the reduction potentials of many Grx proteins vary with GSSG/2GSH concentrations due to their specific interactions [32]. Variations arising from different experimental procedures, conditions and even the protein forms used for the experiments may account for the scattered literature values of $E^{o'}_{P(SS)}$ reported for *Homo sapiens* Grx1 (HsGrx1) and *Escherichia coli* Grx1 (EcGrx1) in Table 1. This may also reflect the dynamic nature of these Grx enzymes as the mechanisms of catalysis can vary with reaction conditions.

Nevertheless, the $E^{o'}_{P(SS)}$ values of dithiol redoxin enzymes are generally associated with their cellular functions. For example, those enzymes acting as reductases of protein disulfides such as Trxs generally possess more negative $E^{o'}_{P(SS)}$ values while those acting as thiol oxidases in oxidizing environments such as PDI and DsbA are usually characterized by more positive $E^{o'}_{P(SS)}$ values (Table 1). Notably, the known reduction potentials of Grxs mostly lie between those of typical reductases and oxidases. Importantly, the reduction potentials of Grxs for their two catalytically competent oxidized forms Grx(SS) and Grx(SH)(SSG) (see below) vary with GSH concentration, enabling Grxs to function as either reductases or oxidases under different cellular conditions [14,32,33].

However, employing $E^{o'}_{P(SS)}$ to predict the thermodynamic activity of Grx enzymes involving GSSG/GSH may be not adequate in many cases, since the glutathionylated form Grx(SSG) could be the effective oxidized form in catalysis, rather than the fully oxidized form Grx(SS). Unfortunately, the useful $E^{o'}_{P(SSG)}$ values

Table 1. pK_a and reduction potentials of certain dithiol proteins that form intramolecular disulfides P(SS) or of monothiol proteins that form mixed disulfides P(SSG)

Enzyme/protein	Organisms	Active site	pK_a	Ref.	$E^{o'}$ (mV) *versus* SHE[a]	Ref.
Dithiols with a redox couple P(SS)/P(SH)$_2$						
Trx1	*S. cerevisiae*	**Cys^{30}**-Gly-Pro-Cys^{33}	–		– 275	[50]
Trx1	*E. coli*	**Cys^{32}**-Gly-Pro-Cys^{35}	7.1	[51]	– 270	[51]
Trx2	*S. cerevisiae*	**Cys^{31}**-Gly-Pro-Cys^{34}	–		– 265	[50]
Trx1	*H. sapiens*	**Cys^{32}**-Gly-Pro-Cys^{35}	6.3	[52]	– 230	[53]
Trx2	*E. coli*	**Cys^{64}**-Gly-Pro-Cys^{67}	5.1	[51]	– 221	[51]
Grx2	*H. sapiens*	**Cys^{37}**-Ser-Tyr-Cys^{40}	4.6	[54]	– 221	[55]
Grx3[b]	*E. coli*	**Cys^{11}**-Pro-Tyr-Cys^{14}	5.2	[56]	– 198	[57]
Grx1	*H. sapiens*	**Cys^{23}**-Pro-Tyr-Cys^{26}	3.5	[30]	– 220	[58]
Grx1-tm[c]	*H. sapiens*	**Cys^{23}**-Pro-Tyr-Cys^{26}			– 232	[55]
					– 169	[32]
					– 118[d]	[31]
Grx1	*E. coli*	**Cys^{11}**-Pro-Tyr-Cys^{14}	< 5.0	[59]	– 233	[57]
					– 168[e]	[32]
PDI	*H. sapiens*	**Cys^{53}**-Gly-His-Cys^{56}	4.81	[60]	~– 165	[61]
DsbA	*Salmonella enterica*	**Cys^{30}**-Pro-His-Cys^{33}	3.3	[62]	– 126	[62]
DsbA	*E. coli*	**Cys^{30}**-Pro-His-Cys^{33}	3.3	[63,64]	– 120	[63,64]
HMA4n	*Arabidopsis thaliana*	Cys^{27}-Cys^{28}	–		– 192	[33]
Atox1	*H. sapiens*	Cys^{12}-Gly-Gly-Cys^{15}	8.9, 5.5[f]	[65]	– 191	[32]
Monothiols with a redox couple P(SSG)/(P(SH) + GSH)						
CopC-H48C	*Pseudomonas syringae*	**Cys^{48}**			– 247	[32]
Grx1-qm[g]	*H. sapiens*	**Cys^{23}**-Pro-Tyr-Ser^{26}			– 230	[32]
Grx1-C14S[e]	*E. coli*	**Cys^{11}**-Pro-Tyr-Ser^{14}			– 213	[32]

[a] For the redox couple P(SS)/P(SH)$_2$ for dithiol proteins (see Eqs. (2a) and (2a')) or the redox couple P(SSG)/(P(SH) + GSH) for monothiol proteins (see Eqs. (1a) and (1a')).
[b] For single mutant Cys65Tyr that retains the two active site Cys residues only.
[c] For triple mutant Cys8,79,83Ser that retains the two active site Cys residues only.
[d] Determined in KPi buffer (pH 7.0) containing [GSH + 2 GSSG] = 1.0 mM.
[e] For a protein form with eight extra residues in the C-terminus derived from a cleavage of the hexa-His tag.
[f] Values for Cys^{12} and Cys^{15}, respectively.
[g] For quadruple mutant Cys8,26,79,83Ser that retains one active site Cys^{23} residue only.

for the redox couple P(SSG)/(P(SH) + GSH) of individual proteins P(SH) remain largely undetermined except for a few monothiol forms reported recently by us [32]. As expected, estimated $E^{o'}_{P(SSG)}$ values fluctuate around the $E^{o'}_{GSSG}$ value with those for the enzymes being somewhat less negative and those for normal protein thiols slightly more negative (Table 1). It must be emphasized that the direction of a redox reaction is determined by the reduction potentials (E') of the related redox couples under specific conditions, not by the standard reduction potentials ($E^{o'}$). Both E'_{GSSG} and $E'_{P(SSG)}$ vary with log [GSH] but the former more sensitively by a factor of 2 (see Eqs. (1a') and (1b')). This allows fine-tuning of the driving force to allow Grxs to catalyze thiol–disulfide exchange reactions in different directions [32,33].

Kinetic considerations

Cellular redox metabolism works under non-equilibrium conditions and is usually controlled by kinetic rather than thermodynamic constraints [66,67]. In this sense, thiol–disulfide oxidoreductases play important roles in facilitating dynamic cellular redox processes. Thiol–disulfide exchange is a second-order reaction that involves sequential bimolecular nucleophilic substitution (S_N2), expressed generally in Eq. (4) [8,20]:

$$RS^-_{nuc} + (RS_c\text{–}S_{lg}R) \rightarrow (RS_{nuc}\text{–}S_cR) + RS^-_{lg} \quad (4)$$

RS^-_{nuc} is the attacking thiolate, RS_c is the central sulfur group that participates in both reactant and product disulfide and RS_{lg} is the leaving group. The attack can only occur along the direction of the disulfide bond, a requirement imposed by the reaction geometry of a trigonal bipyramidal transition state [8,20]. This reaction is slow for aliphatic thiols and disulfides. GSH is a weak nucleophile (due to the high thermodynamic barrier involved in deprotonating its cysteinyl thiol; pK_a ~8.5), GSSG is a weak electrophile and GS is not a good leaving group [29]. Consequently, reactions 1 and 2 involving GSH/GSSG interconversion normally require a redox enzyme [19].

A number of factors contribute to the effectiveness of such an enzyme:

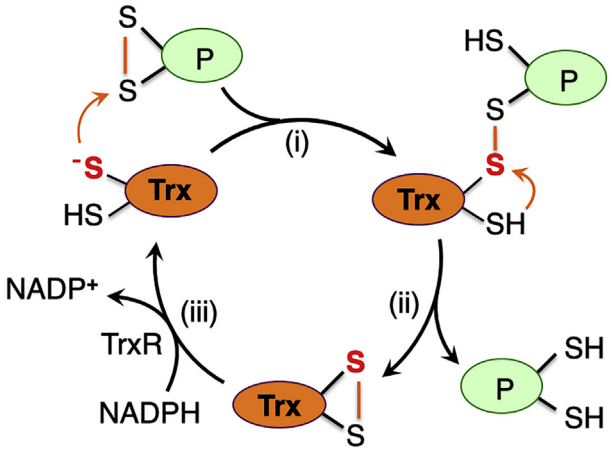
(a) Its reduction potential (not necessarily the standard reduction potential) should lie between those of the redox couples involved in reactions 1 and 2, but of note, the reduction potential may change dynamically with medium conditions, especially for Grx enzymes [32].
(b) It must possess a surface-exposed Cys thiol group with the electron-withdrawing effect on the sulfur atom to be high enough to allow its deprotonation at physiological pH, but ideally low enough (after the deprotonation) for effective nucleophilic attack. Such opposing demands lead to optimized pK_a values just below the solution pH for the catalysis [20,46]. It is important to note that, in the S_N2 transition state, a dynamic pK_a induced by molecular interactions may differ significantly from the static pK_a [68].
(c) It must be a good electrophile for the oxidized form and be a good leaving group at the completion of the catalytic cycle.
(d) It may also possess specific long-range electrostatic interactions and complementary contact surfaces with the protein substrates, especially when the substrate is a protein disulfide [69].

All thiol–disulfide oxidoreductase enzymes possess a surface-exposed Cys thiol with low pK_a (Table 1). This enables them to be effective nucleophiles for the oxidative half-reaction and a good leaving group for the reductive half-reaction [24,70]. In particular, Grxs possess intermediate reduction potentials which also vary with GSH concentration and display specific interaction site (s) for the GS moiety. This enables them to function as a reductase or an oxidase under a variety of cellular conditions with GSH/GSSG as efficient electron donor/acceptor. Consequently, Grxs have evolved as a class of versatile enzymes that directly connect the reversible redox chemistry of protein thiols to the abundant cellular GSSG/GSH thiol pool. After a brief historical perspective, this review will focus on the molecular properties and catalytic mechanisms of the Grx enzymes.

Historical Perspective: Discovery of Thioredoxins

In 1964, *E. coli* Trx1 (EcTrx1) became the first enzyme described as a catalyst for thiol–disulfide exchange and was identified initially as a hydrogen atom donor for the reduction by NADPH of a protein disulfide in the enzyme ribonucleotide reductase (RNR) [71]. RNR catalyzes reduction of each of the ribonucleotides for synthesis of the four building blocks (dNTPs) of DNA and plays a key role in DNA synthesis and repair [72]. Similar properties were ascribed later to EcTrx2 [73]. They possess the most negative reduction potentials among the thioredoxin enzyme family (Table 1) and catalyze reduction of protein disulfides with electrons sourced from NADPH via thioredoxin reductase (TrxR). Scheme 1 depicts the accepted dithiol mechanism.

The active site Cys-xx-Cys motif features a buried C-terminal Cys residue and a solvent-exposed N-terminal Cys whose environment enforces a low pK_a (Table 1). This provides the thiolate function



Scheme 1. Dithiol mechanism for catalytic reduction of a protein disulfide by thioredoxin (Trx). The reduction is coupled to oxidation of NADPH mediated by thioredoxin reductase (TrxR).

that allows nucleophilic attack on a client protein disulfide bond (Scheme 1, step i). Intimate protein-to-protein contact is proposed to enhance this effect by dynamically altering the pK_a of both Cys thiols in the active site [74].

Conformational changes induced in the mixed disulfide product are proposed to activate the nearby internal C-terminal Cys thiol for nucleophilic attack on the N-terminal Cys of the mixed disulfide bond (step ii) [68,75]. In fact, mutation of the C-terminal Cys residue in Trxs prevents step (ii) and has been used to trap and identify protein partners that target Trx [76,77]. A further key aspect is that the close protein-to-protein contact in the mixed disufide prevents attack by external reductants such as GSH, rendering the C-terminal Cys critical to the catalytic cycle. Intermediate Trx(SS) is a direct substrate of TrxR to regenerate the resting enzyme (step iii) [78].

Glutaredoxins

Definition and classification

In the later 1970s, Grxs (also known as "thioltransferases") were differentiated from Trxs as GSH/GSSG-dependent oxidoreductases [79–82]. They are more versatile than Trx enzymes in catalysis, being able to catalyze each of the two types of thiol–disulfide exchange reactions depicted in Fig. 1.

Four Grxs have been identified in *E. coli*, including the classic dithiols EcGrx1 and EcGrx3, a dithiol EcGrx2 of unusually high molar mass and one monothiol EcGrx4 [22]. Four types of *H. sapiens* Grxs (HsGrx1–3 and HsGrx5) have also been identified and are expressed in different cell types [22,27]. HsGrx1 and HsGrx2 have been studied extensively. The former is located primarily in the cytosol but has also been detected in the mitochondrial inter-membrane space and in the nuclei of certain cells [83–85]. It can be exported into the extracellular medium [84,86–90]. Consequently, HsGrx1 assumes diverse catalytic roles in cell signaling and protection [20,27]. HsGrx2 is localized in the mitochondrion while HsGrx3, a multi-domain protein also known as PICOT (PKC-interacting cousin of thioredoxin), is found in both the cytosol and nucleus. They each bind Fe–S clusters and are proposed to serve as redox sensors. HsGrx5 is located in the mitochondrion and is involved in Fe–S cluster assembly [22,27]. Equivalent Grx enzymes appear to be present in many other organisms including Gram-negative bacteria, yeasts and plants [25,26].

Grxs have been classified into two main groups, monothiol or dithiol, based on phylogeny, sequence and domain structure [22]. Most dithiol Grxs contain an active site sequence Cys-Pro-Tyr-Cys (Table 1), whereas monothiol Grxs lack the C-terminal cysteine and feature an active site motif of Cys-Gly-Phe-Ser. The latter may be further divided into single domain enzymes or multi-domain enzymes that contain an N-terminal thioredoxin-like domain and between one and three C-terminal monothiol Grx domains. Additional classification schemes, based on sequence and function, have been proposed [26].

This review focuses on the catalytic mechanism of classic dithiol Grxs including HsGrx1, EcGrx1 and related enzymes. The versatility of their active site allows them to participate in all of the reactions of Fig. 1.

Structures and functions

Most dithiol Grxs are of low molar mass (12–38 kDa) with a thioredoxin fold and a conserved Cys-xx-Cys active site [27]. For example, the structure of HsGrx1 consists of a four-stranded mixed β-sheet surrounded by five α-helices (Fig. 2a) [91]. The Cys^{23}-Pro-Tyr-Cys^{26} active site features a solvent-exposed N-terminal Cys^{23} residue with a low pK_a of ~3.5 and a buried C-terminal Cys^{26} residue with higher pK_a [30]. Reported reduction potentials for the HsGrx(SS)/HsGrx(SH)(S^-) couple vary considerably with GSH/GSSG ratio and concentrations (Table 1), reflecting the dynamic nature of the interactions of Grx with redox partners GSSG/GSH [32].

As discussed above, the pK_a of a thiol group is generally correlated with its reduction potential and determines not only its intrinsic reactivity as an attacking nucleophile but also its reactivity as an electrophile and as a leaving group when present within a disulfide bond [46,47,70]. The very low pK_a of the reactive thiol is expected to (a) increase the reduction potential of the active site, (b) ensure that it is present at physiological pH as a thiolate anion rather than a neutral thiol and so be strongly

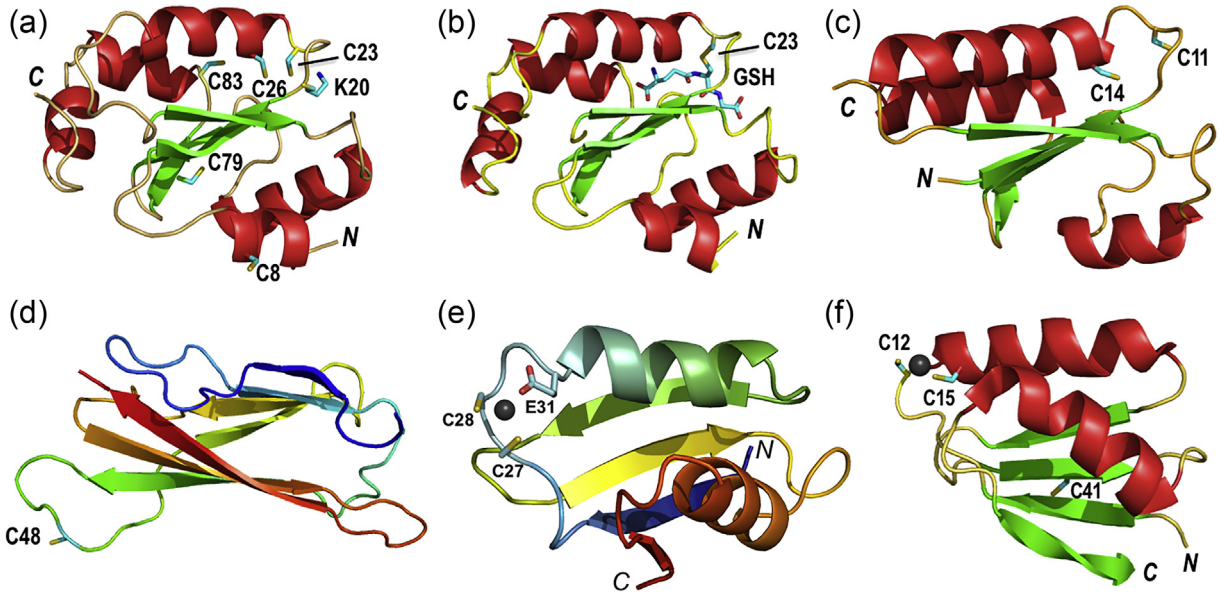


Fig. 2. Protein molecular structures: (a) fully reduced HsGrx1 (PDB code: 1JHB; thioredoxin fold); (b) the glutathionylated HsGrx1-qm (C8,26,79,83S) (1B4Q); (c) fully reduced EcGrx1 (1EGR); (d) PsCopC-H48C model on template PsCopC (2C9Q); (e) Zn^{II}-AtHMA4n (2KKH; ferredoxin fold); and (f) Cu^{I}-Atox1 (1TL4). Labeled amino acid residues and the GSH fragment are shown as sticks while the metal ions in Zn^{II}-AtHMA4n and Cu^{I}-Atox1 are represented as black spheres. Protein N- and C-termini are indicated by N and C, respectively. The structures were generated with PyMOL.

nucleophilic, and (c) increase the electrophilicity and cleavage lability of its disulfide bonds and be a good leaving group upon cleavage.

Grxs feature specific binding site(s) for GSH near their active sites and exhibit a strong substrate preference for GSH/GSSG and P(SSG) [70,92,93]. Structural data of several glutathionylated Grxs are available in which a GS moiety interacts with a group of highly conserved residues and forms a disulfide bond with the surface-exposed N-terminal reactive Cys (Fig. 2b) [34,43,94,95].

Grxs exhibit diverse catalytic functions. The first reaction identified was the catalytic reduction of a protein disulfide in *E. coli* RNR by EcGrx1 at the expense of GSH, an essential step for regeneration of the active form of RNR enzymes [80]. Both Cys thiols in the active site of EcGrx1 are required for the catalysis [43], but similar reduction of a mammalian RNR was accomplished by both HsGrx2 and its C40S variant with equal efficiency [96]. The C40S form retained only a single N-terminal Cys37 in the active site. Oxidized HsGrx2 can accept electrons from either GSH or TrxR [97], but intriguingly, HsGrx2 catalyzed reduction of the mammalian RNR efficiently in the presence of GSH, but not in the presence of TrxR/NADPH, demonstrating that the dithiol mechanism of Scheme 1 is not applicable in this case [96]. On the other hand, in a recent study, a bacterial RNR was demonstrated to be reduced efficiently by a fused Grx with an intact dithiol active site, but a variant form lacking the C-terminal Cys was also active, albeit at a reduced level [45].

Grxs from a variety of organisms are effective in catalyzing deglutathionylation of a range of mixed disulfides [92,93,98]. Two distinct glutathione interaction sites appear to be required for efficient catalysis [23]. HsGrx1 was demonstrated to mediate both oxidation and reduction of the copper metallochaperone Atox1 (Fig. 2f) using GSSG/GSH as redox cofactors, under both *in vivo* and *in vitro* conditions [31,58]. It also catalyzes reduction of a protein disulfide bond in *H. sapiens* Cu,Zn superoxide dismutase (HsSOD1) at the expense of GSH [35]. Both HsGrx1 and EcGrx1 catalyze reduction of a non-active-site protein disulfide bond between Cys62/Cys69 in *H. sapiens* HsTrx1 [36]. The presence of this bond inactivates HsTrx1. In concert with a disulfide-isomerase, EcGrx1 catalyzes GSH-dependent folding of reduced ribonuclease [99].

HsGrx2 catalyzes reversible oxidation and glutathionylation of mitochondrial membrane thiol proteins, and also reduction of protein disulfides and deglutathionylation of mixed disulfides [54,97,100]. More recently, both HsGrx1 and EcGrx1 were shown to catalyze each of the thiol–disulfide exchange reactions shown in Fig. 1 in both directions on several model substrates, the monothiol protein CopC-H48C and the dithiol proteins Atox1 or domain HMA4n (Fig. 2d–f) [31–33].

Several mechanisms have been proposed to interpret the diverse catalytic reactions of Grxs observed under the different conditions. However, confusion and controversies remain, as highlighted above for the catalytic reduction of RNR by Grxs/GSH under different

conditions. We now discuss these reaction mechanisms systematically.

Catalytic Mechanisms

General considerations

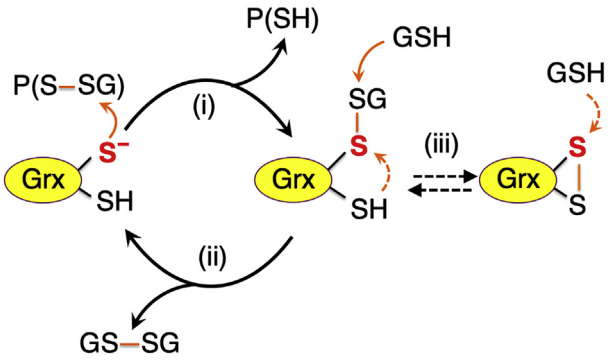
As mentioned above, Grxs are capable of catalyzing each type of reaction shown in Fig. 1. The overall direction depends not only on the reduction potential of the GSSG/2GSH couple but on the nature of the protein thiols involved and on other solution conditions such as the presence of metal ions. During catalysis, Grxs are demonstrated to shuttle *rapidly* between three oxidation states: dithiol Grx(SH)(S^-) with a deprotonated N-terminal Cys thiolate, mixed disulfide Grx(SH)(SSG), and disulfide Grx(SS) [23,32,33]. The particular reaction mechanism employed may differ from case to case.

Mutation of individual Cys residues in the active site has been instructive in characterizing catalytic intermediates [34,43]. However, the existing literature emphasizes the catalytic role of Grxs in deglutationylation of mixed disulfides with lesser or no consideration of other types of reactions. In addition, the proposed mechanisms are often speculative and controversial. A significant challenge for mechanistic study is the lack of research tools that allow reliable speciation analysis along the reaction time course. Recently, we demonstrated that quenching of the reactions with excess iodoacetamide followed by protein speciation analysis via ESI-MS allowed interception and characterization of *both* substrate *and* enzyme intermediates and provided new insights into the reaction mechanisms [31–33].

Reduction of glutathionylated disulfides

The forward reaction of Fig. 1a (Eq. (1)) is the "deglutathionylation" of protein disulfides P(SSG) by GSH, widely believed to be the main function of Grxs. Both GSH and P(SH) are aliphatic monothiols with similar pK_a (both ~8.5) and so the thermodynamic stabilities of P(SSG) and GSSG relative to their respective fully reduced forms should be comparable (i.e., $E^{o'}_{P(SSG)} \approx E^{o'}_{GSSG}$; see Eq. (1')). This was confirmed for the monothiol protein CopC-H48C(SH) (Table 1; Fig. 2d). Therefore, according to Eq. (1'), the equilibrium position of P(SSG)/P(SH) in Eq. (1) should be governed primarily by the GSSG/GSH molar ratio. The concentration of GSSG in the cytosol is extremely low [13,14], indicating that the forward reaction of deglutathionylation is strongly favored thermodynamically.

Many disulfides P(SSG) are reduced by Grx/GSH via a so-called ping-pong mechanism (Scheme 2) [32,34,92,93]. A catalytic cycle starts with nucleophilic attack of the N-terminal Cys thiolate of Grx on the client disulfide P(SSG) to release the protein thiol P(SH) and to yield an enzyme intermediate Grx(SH)(SSG) (step i). The latter is unstable to external attack by GSH, regenerating the resting form Grx(SH)(S^-) (step ii). It is also susceptible to *competitive* internal attack by the C-terminal Cys thiol to yield an inhibited disulfide form Grx(SS) (step iii). However, step (iii) is fast and reversible [32,33,101]. Consequently, although Grx(SS) is usually more stable than Grx(SH)(SSG), it is the latter that plays a direct role in this catalysis (but the former may have a direct role in reduction of protein disulfides, see below) [31–33]. The catalytic process proceeds effectively via a monothiol mechanism comprising the two separate redox processes: the oxidative half-reaction of step (i) and the reductive half-reaction of step (ii).



Scheme 2. Proposed ping-pong mechanism for deglutathionylation of a protein glutathionyl disulfide P(SSG) by Grx/GSH [32,34]. For intermediate Grx(SH)(SSG), competing nucleophilic attack by GSH and the C-terminal Cys thiol on the mixed disulfide bond leads to either regeneration of the active enzyme Grx(SH)(S^-) or formation of the internal disulfide Grx(SS). Dashed lines indicate non-catalytic processes, and disulfide bonds are shown in orange. Reproduced with permission from Ref. [32].

Steps (i) and (ii) identify the Grx enzyme substrates as P(SSG) and GSH, respectively. The requirement for a trigonal bipyramidal reaction geometry for the central sulfur atom in each transition state (see Eq. (4)) means that two distinct GS interaction sites may be required for *efficient* catalysis: one for P(SSG) and the other for GSH. Consequently, Deponte proposed two models for Grx catalysis (see Fig. 3) [8,67,102]: one is the glutathione scaffold model in which a scaffold site in Grx serves as a binding site for the GS moiety of the disulfide substrate P(SSG) and facilitates step (i) of Scheme 2; the other is the glutathione activator model in which a defined site in Grx binds GSH and promotes step (ii), as suggested also in a previous study [70]. These two models are not necessarily exclusive. For example, a conformational change of the scaffold model after step (i) may allow the reductive half-reaction to proceed via an activator model with GSH

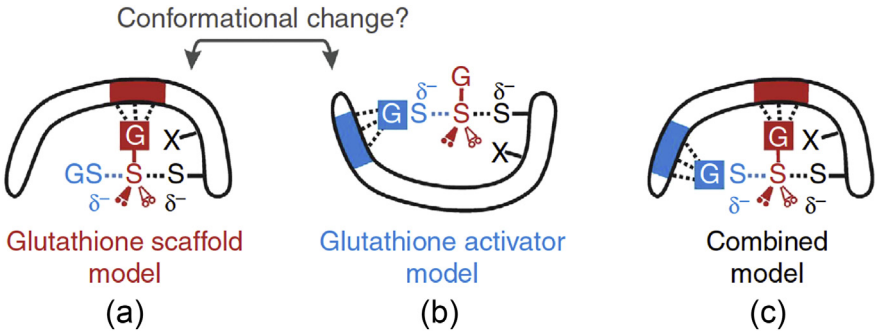


Fig. 3. Refined models for Grx catalysis: (a) glutathione scaffold model for which the glutathionyl moiety of disulfide substrate PSSG or GSSG occupies the glutathione binding site during the entire catalytic cycle; (b) glutathione activator model for which interaction between Grx and GSH for the reductive half-reaction plays the most important role in catalysis; and (c) combined scaffold-activator model that requires the presence of two distinct glutathione-interaction sites in the Grx enzyme. Reproduced with permission from Ref. [23].

substitution of the GS moiety of P(SSG). Then, the scaffold site and the activator site would be identical (Fig. 3a, b). Alternatively, a Grx enzyme may contain two distinct sites for efficient redox catalysis, one acting as the scaffold site and the other as the activator site (Fig. 3c). The latter case was demonstrated recently for the monothiol ScGrx7 from *Saccharomyces cerevisiae* [23].

Consistent with the scaffold model, dithiol Grxs from various sources were shown to exhibit a strong substrate preference for substrate P(SSG) in step (i) [98], attributable to recognition of specific GS binding [103]. Mutation of the residues of the GS binding site in EcGrx1 led to significant loss of activity for a model substrate [98]. With P(SSG) as substrates, enhanced accessibility to the glutathionylated site in Grxs and the low pK_a of the Cys residue were identified as the main determinants of substrate specificity [103].

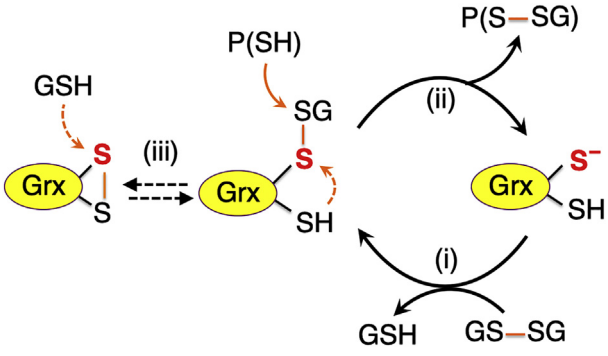
Mutation of the C-terminal Cys to Ser in dithiol Grxs generally leads to a significant increase in enzyme activity, consistent with elimination of inhibitory exchange step (iii) [32,98]. Here the C-terminal Cys thiol appears to act as a "catalytic brake" for the catalysis. However, intriguingly, most monothiol Grxs lack GSH-dependent thiol–disulfide exchange activity and this was ascribed to a lack of a specific binding site for the GS moiety [23,104]. For example, monothiols ScGrx3/4 are unable to catalyze deglutathionylation of a glutathionyl mixed disulfide using GSH as reductant: excess proteins were required to effect the reduction [105].

Oxidation of protein monothiols

This is the reverse reaction of Fig. 1a (Eq. (1)) that produces a mixed disulfide P(SSG). The simplest mechanism is that depicted in Scheme 3. Note that step (ii) here is related to step (ii) in Scheme 2. However, Grx(SH)(SSG) interacts with P(SH) rather than GSH and displays no substrate preference for GSH over other reducing thiols, at least for the human and yeast Grx1s [98]. Step (ii) in Scheme 3 is plausible since the "normal" thiol in P(SH) (pK_a of ~8.5) is expected to be a less effective nucleophile for attack on the stable disulfide bond in GSSG (the second reactant) than for attack on the more labile mixed disulfide bond in Grx(SH)(SSG). In addition, step (ii) is promoted by the good leaving properties of Grx(SH)(S^-) [32,70].

However, this scenario is challenged by at least three potentially competing reactions or barriers: (a) formation of the internal Grx(SS) disulfide via step (iii) that, fortunately, is reversible; (b) reversal of step (i) induced by external GSH present at high relative concentration; this again is reversible; and (c) possible electrostatic and/or steric barriers for direct protein–protein contact between Grx and P(SH). Therefore, to enforce process (ii), a high GSSG concentration is required to convert Grx(SH)(S^-) optimally to the active enzyme form Grx(SH)(SSG).

We documented the latter by demonstrating that both HsGrx1 and EcGrx1 can catalyze direct attack by



Scheme 3. Proposed ping-pong mechanism for glutathionylation of protein monothiol P(SH) by Grx/GSSG. Dashed lines indicate non-catalytic processes. The reactive sulfur atoms are shown in red, and the disulfide bonds involved in exchange are shown in orange. Reproduced with permission from Ref. [32].

GSSG on the solvent-exposed thiol in CopC-H48C (SH) by GSSG (Fig. 2d) [32]. However, the extent of reaction was dependent on GSSG/GSH, consistent with the assumption that $E^{o'}_{P(SSG)} \approx E^{o'}_{GSSG}$ for two aliphatic monothiols P(SH) and GSH (Eqs. (1a) and (1b)), confirmed in this case for the CopC monothiol (see Table 1) [32].

On the other hand, it has been confirmed that it is more difficult to glutathionylate the monothiol Cys^{41} in Atox1 that is buried within a panel of four rigid β-sheets (Fig. 2f) and which does not have ready access to the reaction site in HsGrx1. In contrast, the surface-exposed Cys^{12}-xx-Cys^{15} dithiol in Atox1 can be oxidized readily via a sequential glutathionylation-deglutathionylation reaction process (vide infra) [31]. Consequently, catalytic *S*-glutathionylation of protein monothiol P(SH) by Grxs at the expense of GSSG (Scheme 3) can only be significant for those protein thiols that are accessible in an oxidizing environment with a relative high GSSG/GSH ratio. These may include those in (a) the mitochondrial membrane (where protein thiols are oxidized catalytically by HsGrx2) [100]; (b) the *trans*-Golgi network (TGN; where GSSG/GSH ratios could reach 1:1 and where many nascent proteins are processed for secretion) [15,106]; (c) cellular vacuoles into which GSSG is actively transported [13]; and (d) extracellular milieux where the ratio of the secreted GSSG/GSH may be elevated [16,17]. Thermodynamically, direct protein *S*-glutathionylation is less likely to occur in cytosols where GSSG concentrations are extremely low [24].

However, protein *S*-glutathionylation may occur spontaneously with less discrimination via indirect pathways involving reactive intermediates such as sulfenic acids (Eqs. (5) and (6)), sulfenyl amides (Eq. (7)), thiyl radicals (Eq. (8)) and thiosulfinates (Eq. (9)) [9,107,108]:

$$P(SOH) + GSH \rightarrow P(SSG) + H_2O \quad (5)$$

$$G(SOH) + P(SH) \rightarrow P(SSG) + H_2O \quad (6)$$

$$PS(NH)(CO)R + GSH \rightarrow P(SSG) + H_2N(CO)R \quad (7)$$

$$PS\bullet + GS\bullet \rightarrow P(SSG) \quad (8)$$

$$PS(O)SP + GSH \rightarrow P(SSG) + P(SOH) \quad (9)$$

These intermediates may be generated even in reducing cytosols via direct oxidation of protein thiols by ROS and may have important roles in cellular redox signaling. Interestingly, protein *S*-glutathionylation occurs via reduction of these reactive protein intermediate species by GSH, not by direct *S*-glutathionylation of their protein thiols.

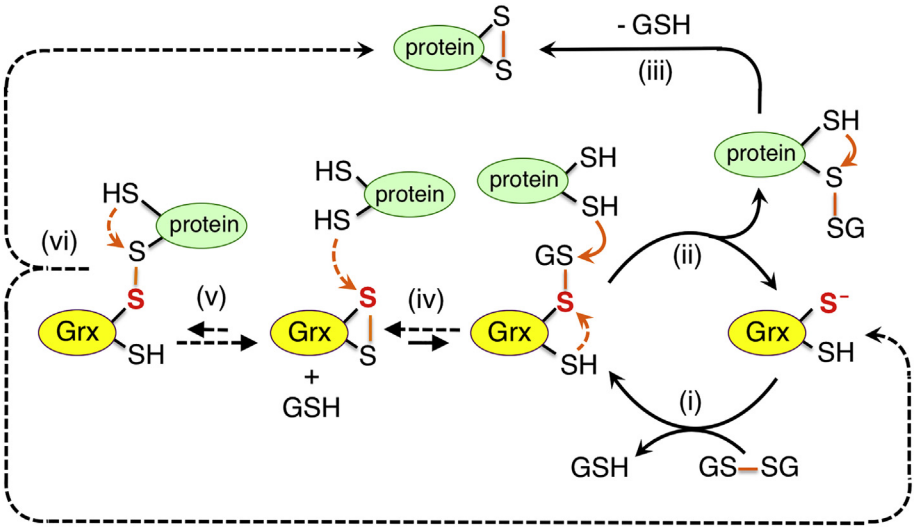
Common features for reversible *S*-glutathionylation

Mutation of the C-terminal Cys to Ser in dithiol Grxs generally leads to a 2- to 4-fold increase in activity for *S*-glutathionylation or deglutathionylation, but the equivalent mutation of the N-terminal Cys residue results in complete loss of activity [32,34,98]. This suggests that catalysis relies on the single N-terminal Cys residue only and that the C-terminal Cys residue acts essentially as a "catalytic brake" via inhibitory exchange step (iii) (see Schemes 2 and 3). However, intriguingly, most monothiol Grxs lack GSH-dependent thiol–disulfide exchange activity and this was ascribed to a lack of a specific binding site for the GS moiety [23,105].

The enzyme intermediate Grx(SH)(SSG) proposed in both Schemes 2 and 3 is unstable but was detected as a trace component in fast equilibrium with Grx(SS) (step (iii) in both schemes) [31–33]. It was stabilized and characterized also upon mutation of the C-terminal Cys of the Cys-xx-Cys motif to Ser [34,43]. For the same Grx protein, $E^{o'}_{P(SSG)}$ is about 50 mV more negative than $E^{o'}_{P(SS)}$ (Table 1) [32], but under specific conditions, it is the half-cell reduction potential E', not the corresponding standard potential $E^{o'}$, that determines the redox states of related thiol–disulfide components. $E'_{P(SSG)}$ is dependent on GSH concentration via ln [GSH], while $E'_{P(SS)}$ is not (Eq. (1a) *versus* Eq. (2a)). It is estimated from the two Nernst equations that $E'_{P(SSG)}$ is more positive than $E'_{P(SS)}$ at [GSH] < 50 mM. This would explain why Grx(SH)(SSG) is usually not stable under normal cellular conditions and undergoes spontaneous exchange to yield Grx(SS) and GSH. In fact, this spontaneous reaction inhibits the catalytic enzyme activity of Grxs [32].

Oxidation of protein dithiols

This is the reverse reaction of Fig. 1b (Eq. (2)) in which the monothiol substrate P(SH) in Scheme 3 is replaced by a protein dithiol $P(SH)_2$ with two vicinal cysteinyl thiols that are oxidized readily to disulfide P(SS). Examples include many metal-binding proteins or domains such as $Atox1(SH)_2$ and $HMA4n(SH)_2$ (Fig. 2e, f) [31,33]. We demonstrated that oxidation of $HMA4n(SH)_2$ by Grx/GSSG proceeds via parallel monothiol–dithiol mechanisms, as shown in Scheme 4 [33]. Oxidation via the monothiol route (in solid arrows) follows essentially that for monothiol substrate P(SH) of Scheme 3, but the glutathionylated form P(SH)(SSG) is unstable and is converted, via *spontaneous* chemical step (iii), to the final product P(SS) [31,32]. Grx(SH)(SSG) is also converted spontaneously to more stable Grx(SS) (step (iv)), but the difference is that the latter can be rapidly converted by GSH back to Grx(SH)(SSG) via reversible step (iv) while the normal



Scheme 4. Proposed parallel monothiol–dithiol mechanism for catalytic oxidation of protein dithiol $P(SH)_2$ (such as $HMA4n(SH)_2$) by Grx/GSSG. The monothiol oxidation route is shown in solid lines, whereas the dithiol oxidation route is shown in dashed lines. Under the oxidative conditions, the resting enzyme forms for dithiol Grx and monothiol Grx are Grx(SS) and Grx(SSG), respectively. Adapted after Ref. [33].

protein disulfide P(SS) cannot (see further discussion below) [32,33].

Under oxidative conditions, we found the resting enzyme form to be Grx(SS). This was able to catalyze $P(SH)_2$ oxidation by a parallel dithiol mechanism (in dashed arrows) via a transient disulfide complex Grx(SH)(SS)(SH)P [33]. We observed that (1) in the absence of GSH, thiol–disulfide exchange between proteins P1 and P2 (in respective dithiol $P1(SH)_2$ and disulfide P2(SS) forms) is catalyzed by dithiol $Grx(SH)(S^-)$ but not by monothiol variant $Grx(OH)(S^-)$ that lacks the C-terminal Cys residue in the active site (Scheme 5a), and (2) addition of trace GSH rescues the catalytic activity of $Grx(OH)(S^-)$ according to Scheme 5b. Consequently, catalytic oxidation of protein dithiol $P(SH)_2$ by Grx/GSSG can proceed via distinct dithiol and monothiol mechanisms, as depicted in Scheme 4 [33]. Substitution of the C-terminal Cys residue with Ser in $Grx(SH)(S^-)$ prevented formation of the disulfide Grx(SS), eliminating the possibility of protein dithiol oxidation via the dithiol route (iv) → (v) → (vi). This led to doubling of the Grx activity for the catalytic oxidation of $HMA4n(SH)_2$ (Fig. 2e), demonstrating that the monothiol route is more efficient than the dithiol route in this case [33].

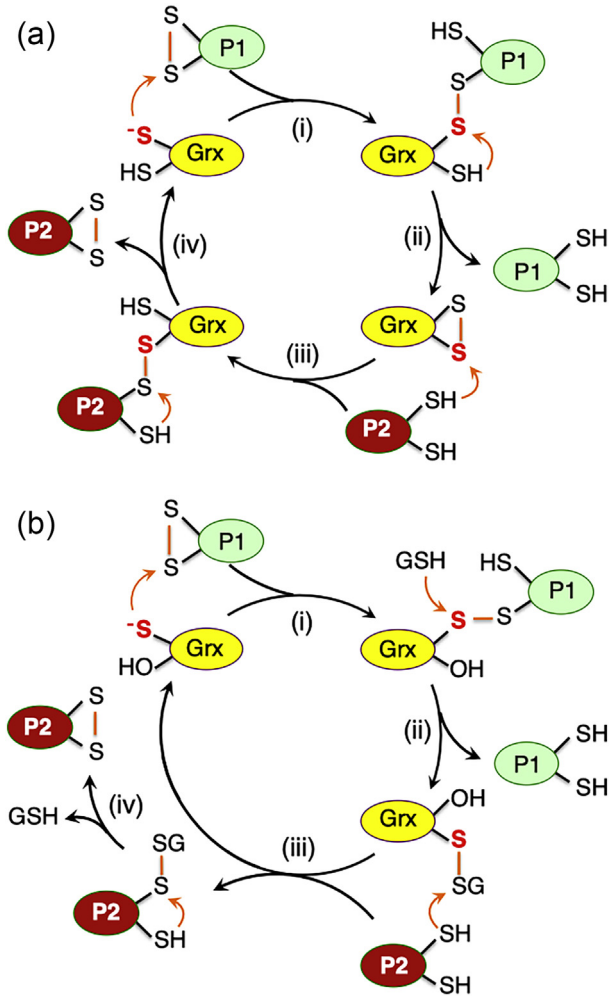
Protein dithiols $P(SH)_2$ including $Grx(SH)(S^-)$ can frequently bind metal ions with high affinity (Fig. 1b; Cu(I) and Zn(II) in particular) [31,109,110]. Metal-binding to the dithiol site stabilizes the reduced form and inhibits Grx catalytic activity [31]. Consequently, the reactions in Scheme 4 are inhibited by the presence of transition metal ions [31].

The oxidative half-reaction of step (i) in Scheme 4 is the same as step (i) in Scheme 3 and may be understood by the glutathione scaffold model of Fig. 3a. However, the reductants in reaction Schemes 3 and 4 are protein monothiols P(SH) and dithiols $P(SH)_2$, respectively, not GSH. Consequently, the glutathione activator model (see Fig. 3b) is not applicable in these cases.

The reactions defined by Scheme 4, as the case for Scheme 3, may be more relevant in oxidized cellular compartments, but less likely in highly reduced cytosols. In particular, full oxidation of one equivalent of protein dithiol $P(SH)_2$ to disulfide P(SS) converts one equivalent of GSSG to two equivalents of GSH and thus the oxidation is driven not only by the high molar ratio of GSSG/GSH, but also by the overall low concentrations of GSSG and GSH such as those in an extracellular environment [16,17]. HsGrx1 was detected at higher levels in the cerebrospinal fluid of patients with Alzheimer's disease and in plasma of patients with diabetes or chronic kidney disease [87–90]. These observations indicate elevated oxidative stress in these patients and suggest that, in extracellular environments, HsGrx1 is more actively involved in protective oxidation of protein thiols possibly via the reactions shown in Schemes 3 and 4.

Reduction of protein disulfides

This is the reverse of dithiol oxidation and is driven thermodynamically by elevated GSH concentrations such as in a highly reducing cytosolic environment (see Eq. (2) and Fig. 1b). Logically, catalysis by a Grx enzyme may proceed via each of the reverse processes given in Scheme 4, and this leads to the possibility of different mechanisms that are currently being debated, as discussed below.



Scheme 5. Dithiol mechanism in the absence of GSH (a) and monothiol mechanism in the presence of GSH (b) employed by Grx enzymes catalyzing thiol–disulfide exchange between proteins P1(SS) and P2$(SH)_2$. Reproduced with permission from Ref. [33].

Monothiol mechanism with a pre-equilibrium glutathionylation step

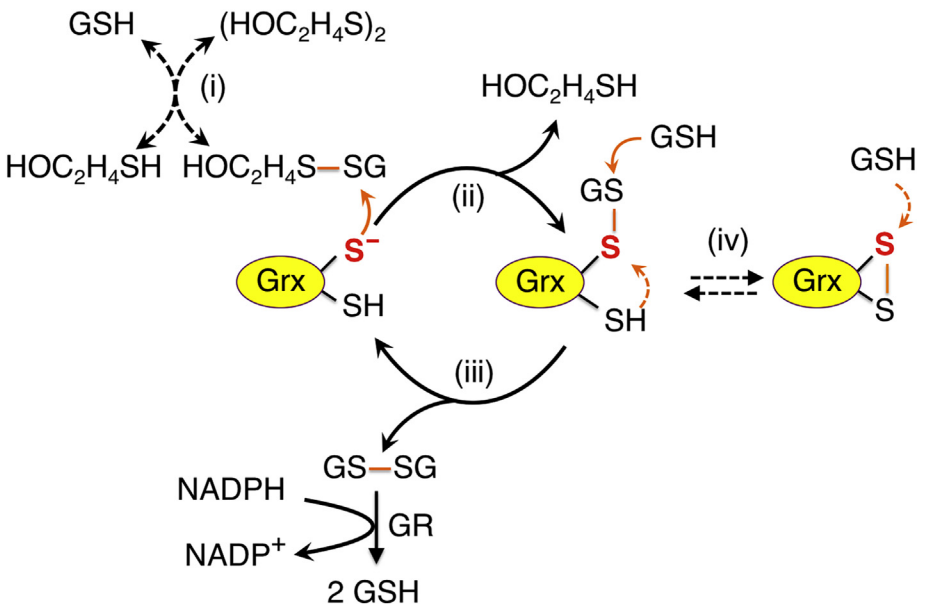
This was proposed initially for the catalytic reduction of model substrate bis(2-hydroxyethyl)disulfide (HEDS), a classic assay for Grx activity that is shown in Scheme 6 [43,92]. Substrate reduction is coupled to reduction of product GSSG by glutathione reductase (GR) at the expense of chromophoric reductant NADPH [111]. Since Grx enzymes prefer glutathionyl disulfides as substrates, a pre-equilibrium involving HEDS and GSH was assumed to generate the actual substrate $HO(CH_2)_2SSG$ (step i) that was reduced by Grx to product $HO(CH_2)_2SH$ (step ii). The ping-pong mechanism is completed via reduction of enzyme by a second equivalent of GSH (step iii).

This mechanism has been used to rationalize reduction of certain protein disulfides by Grx/GSH [14,35,36,96]. In fact, it is equivalent to a proposal that reduction of protein disulfide P(SS) at elevated GSH concentration may follow a reverse route of (iii) → (ii) → (i) in Scheme 4. However, we have demonstrated that the key reverse step (iii) (that can occur at elevated GSH concentrations) is kinetically unfavorable and adjusts too slowly to account for the observed enzyme activity [33]. This could be attributed to (1) the poor nucleophilicity of GSH at physiological pH and (2) the lack of a specific GSH binding site on a typical protein disulfide P(SS). A recent re-evaluation of the reaction mechanism of the HEDS assay led to similar conclusions and suggested that the reaction is most plausibly interpreted as a Grx-catalyzed direct reduction of HEDS [112], as was also suggested in an earlier study [113].

Relevant observations were provided from studies of the superoxide dismutase HsSOD1 that examined reduction of an intramolecular disulfide bond that plays a key role in maintaining enzyme stability and quaternary structure [114]. It was demonstrated that (1) the disulfide bond is a substrate of HsGrx1 and is reduced more slowly in the native enzyme than in a variant form that induces the disease amyotrophic lateral sclerosis (ALS), and (2) the reduction proceeds via a monothiol mechanism that requires only the N-terminal active site Cys^{23} residue of HsGrx1 [35]. It was assumed that the reaction proceeds via a GSH pre-equilibrium monothiol mechanism such as that depicted in Scheme 6 [35]. If this is the case, the putative disulfide HsSOD1(SH)(SSG) should have accumulated and been detected in Grx-deleted cells. However, this was not seen [115].

In a related case, HsTrx1 was inactivated by formation of a disulfide bond between Cys^{62} and Cys^{69} that are not involved in its dithiol active site. The enzyme could be reactivated by reduction of this bond by HsGrx1 or EcGrx1 in the presence of GSH [36]. The reactivation was shown to involve a monothiol mechanism and this was again assumed to proceed via the pre-equilibrium glutathionylation mechanism [36]. However, there is a problem with this interpretation: the pre-equilibrium is expected to cleave the inhibiting $Cys^{62}(SS)Cys^{69}$ disulfide bond to generate a mixed disulfide P(SH)(SSG) that, as discussed above, is generally more reducible chemically than the original protein disulfide P(SS) at [GSH] < 50 mM and then the inhibited HsTrx1 should be re-activated with no need of the second step of enzymatic reduction. However, the Grx enzymes in combination with GSH were required to re-activate the catalytic function of HsTrx1 [36].

A similar scenario is the monothiol mechanism mentioned above for the catalytic reduction of mammalian RNR by HsGrx2/GSH that was interpreted also to proceed via the glutathionylation mechanism of Scheme 6 [96]. If this was the case, GSH alone should be able to induce some residual RNR activity for the same reason. However, again,



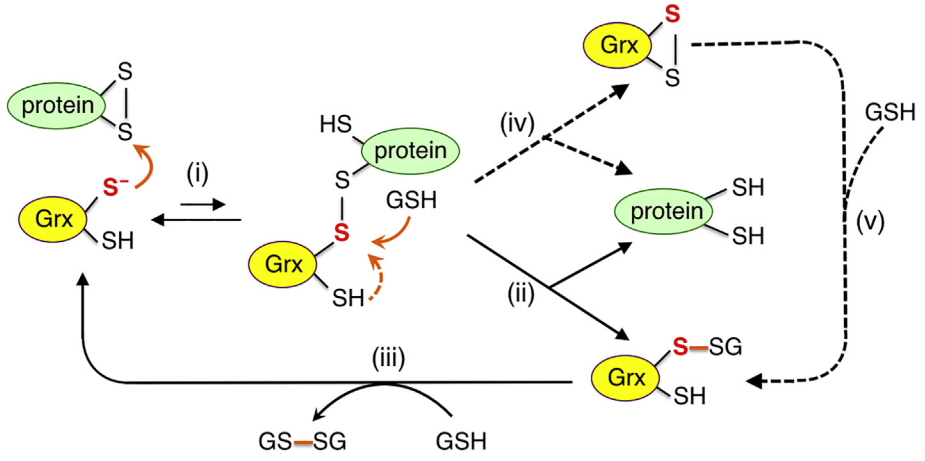
Scheme 6. Putative chemical pre-equilibrium monothiol mechanism for HEDS assay [43,92]. Dashed lines refer to non-catalytic processes.

HsGrx2 was required for the activity of RNR in addition to GSH [96].

Parallel monothiol–dithiol mechanisms

In such cases, the reverse of step (vi) of Scheme 4 leads to alternative mechanisms for catalytic reduction of disulfide P(SS) by Grx/GSH that are depicted by Scheme 7. Dithiol Grxs are adapted to catalyze reduction of disulfides P(SS) by GSH via direct attack on their disulfide bonds to form an enzyme–protein disulfide complex (step i). This can be followed by either a monothiol mechanism (steps ii and iii) or a dithiol mechanism (step iv) or both in parallel [31,33].

This reaction scheme was proposed initially from our study of the catalytic reduction of the protein disulfide Atox1(SS) by HsGrx1/GSH and supported by direct detection of the key intermediate species Atox1(SH)(SS)(SH)Grx by ESI-MS [31]. The reduction, as anticipated, is promoted by the availability of Cu(I) which binds and stabilizes the reduced form [31]. *In vivo* experiments have demonstrated that GSH can reduce Atox1(SS) to allow Cu(I) binding



Scheme 7. Proposed parallel monothiol–dithiol mechanism for catalytic reduction of protein disulfides P(SS) (such as HMA4n(SS)) by Grx/GSH. The monothiol reduction route is shown in solid arrows with the dithiol route in dashed arrows. Under the reducing conditions, the resting forms for dithiol Grx and monothiol Grx enzymes are $Grx(SH)_2$ and Grx(OH)(SH), respectively. Adapted after Ref. [33].

and that HsGrx1 is involved [58]. This reaction scheme was elaborated in our subsequent study of catalytic reduction of a model substrate HMA4n(SS) with the two representative dithiol Grx enzymes HsGrx1 and EcGrx1 [33].

Step (ii) is the key for the monothiol reduction route in which the protein mixed disulfide is attacked by an external GSH molecule to complete the reduction and to generate the glutathionylated enzyme Grx(SH)(SSG). This proposal is supported by the confirmed step (ii) of reaction Scheme 5b. On the other hand, step (i) followed by step (iv) constitutes a dithiol mechanism that was originally proposed by Bushweller *et al.* [43] in their study of reduction of RNR by EcGrx1/GSH (see further discussion in the following section on the dithiol mechanism) and is supported by the confirmed step (ii) of reaction Scheme 5a. Both Grx(SH)(SSG) and Grx(SS) generated via the two mechanisms are converted to the resting enzyme form Grx(SH)(S^-) readily under the reducing conditions imposed by high GSH concentrations [32].

The key step (ii) of the monothiol mechanism is promoted by the specific interaction between GSH and Grx (Figs. 2b and 3b) and by the high concentration of GSH, whereas the key step (iv) of the dithiol mechanism is independent of GSH concentration. Consequently, the relative contributions of these routes to the overall reduction of a protein disulfide may vary with cellular conditions and compartments.

Mutation of the C-terminal Cys to Ser in both HsGrx1 and EcGrx1 led to ~17% increase in their catalytic activities for reduction of HMA4n(SS) by GSH [33]. This is consistent with the parallel operation of both monothiol and dithiol mechanisms and confirms the catalytic brake effect of the C-terminal Cys residue since it allows for formation of less reactive Grx(SS). On the other hand, the rate of reduction of HMA4n(SS) was much slower than that for oxidation of $HMA4n(SH)_2$ by the same Grx enzyme (by ≥75%) [33]. This underscores the difference in the forward and back reaction rates of chemical step (iii) in Scheme 4.

While Grx enzymes generally prefer glutathionyl disulfides P(SSG) as substrates due to the favorable enzyme-substrate interactions (Fig. 3) [23,43,92,98], it is apparent that the active thiolate in Grx(SH)(S^-) may, under the right conditions, attack non-glutathionyl protein disulfides directly, as is the case for thioredoxins Trx(SH)(S^-) (see Schemes 5 and 7 *versus* Scheme 1). The reactive thiolate in Grxs and at least one Cys thiol in Atox1 and HMA4n are surface-exposed (Fig. 2), and this provides steric accessibility for direct protein–protein contact to form the mixed disulfide intermediate (step (i)). It also allows for subsequent access for an external GSH to effect the monothiol reduction step (ii), in addition to the dithiol reduction step (iv).

These parallel monothiol–dithiol mechanisms may have general implications for reduction of protein disulfides P(SS) by GSH that are catalyzed by Grxs that feature a dispensable C-terminal Cys residue. Certainly, the several examples of protein disulfide reduction by Grx/GSH given above may all be interpreted to proceed via the parallel monothiol–dithiol mechanisms of Scheme 4 with a case-dependent contribution from each mechanism. A recent study has confirmed this situation for catalytic reduction of a bacterial RNR by its fused Grx at the expense of GSH: the dithiol route was shown to be more efficient [45]. Intriguingly, similar reduction of a mammalian RNR by HsGrx2 was shown to proceed via the monothiol route exclusively while an equivalent reduction of *E. coli* RNR by EcGrx1 used the dithiol route only [43,96]. The molecular basis for these differences has yet to be explored (but see discussion below on the dithiol mechanism).

Dithiol mechanism

Both active site Cys residues in EcGrx1 have been reported to be essential for catalytic reduction of certain protein disulfides. For example, mutation of the C-terminal Cys^{14} to Ser in EcGrx1 led to complete loss of activity for reduction of a protein disulfide bond in RNR or in bovine pancreas insulin [43,116]. This is likely due to limited access of the external GSH to the EcGrx1-protein disulfide bond. Indeed, an NMR structural study of catalytic GSH reduction of RNR revealed that a disulfide bond was formed between the EcGrx1-C14S variant and a peptide of the interacting subunit B1 [117]. The B1 peptide occupied the GSH binding site in EcGrx1 and protected the disulfide bond from attack by an external nucleophile. Consequently, the key monothiol step (ii) in Scheme 7 is blocked and the reduction can proceed only via the dithiol route of (i) → (iv) → (v) → (iii). This route is similar to the dithiol mechanism employed by Trx enzymes, but differs in regeneration of the respective resting enzyme forms Trx(SH)(S^-) and Grx(SH)(S^-) from their disulfide forms. The former relies on TrxR enzyme with NADPH as reductant whereas the latter is generated directly by GSH (see Scheme 1 *versus* Scheme 7).

A similar example is provided by an elegant study of the enzyme *E. coli* 30-phosphoadenosine 50-phosphosulfate (PAPS) reductase [44]. A disulfide bond forms between the single active site thiols of two monomers as a consequence of the catalyzed reduction of PAPS substrate [44,69]. This bond can be reduced by all Trx/NADPH systems tested but only by a few Grx/GSH systems including EcGrx1/GSH. Most other Grx enzymes were not effective [69]. Mutation of the C-terminal Cys^{14} to Ser in EcGrx1 led to almost complete loss of the activity [44]. It is apparent that neither the GSH pre-equilibrium monothiol mechanism

(Scheme 6) nor the monothiol mechanism via the key step (ii) in Scheme 7 can be applied in this case. The reaction must proceed via a dithiol mechanism as discussed above.

Possible Roles for the C-Terminal Active Site Cys Thiol

Grx catalysis generally proceeds via a monothiol mechanism relying on the N-terminal Cys thiolate only (Schemes 2–7) although a parallel dithiol mechanism can contribute (Schemes 4, 5a, and 7). What, then, is the other role(s) of the C-terminal Cys thiol in Grx enzymes [33].

Certainly, the C-terminal Cys thiol promotes formation of the less reactive disulfide form Grx(SS) during catalysis and acts formally as a catalytic brake. However, there are certain cases in which the intimate enzyme-substrate interaction prevents access of an external GSH to the mixed disulfide bond to assist the reductive half-reaction via the monothiol mechanism (i.e., step (ii) in Scheme 7). Then the catalysis must proceed via further enzyme oxidation involving the C-terminal Cys thiol (i.e., step (iv) in Scheme 7).

Another potential function is to provide a metal ion binding site Cys-xx-Cys with the N-terminal Cys residue. This sequence is present in many metal-trafficking proteins [118,119]. Although a *cis*-proline residue within and/or near this motif in Grxs and Trxs was predicted to inhibit metal ion binding [120], it has now been demonstrated that the active site motif in both HsGrx1 and EcTrx binds Cu(I) with femtomolar affinity at pH 7.0 [31,38]. Both Cys residues are required for high affinity binding, but at pH 7.0, the affinities are about 2 orders of magnitude weaker than those of similar Cys-xx-Cys motifs in typical copper transporting proteins such as Atox1 [31,38]. This can be attributed to the low pK_a of the N-terminal Cys thiolate.

Cu(I) binding to HsGrx1 blocks the active site Cys thiolate and inhibits enzyme activity for all types of thiol–disulfide exchange reactions [31]. This may provide a mechanism for regulation of enzyme activity that is dependent upon reduction potential and copper availability. Interestingly, the enzyme activity of mitochondrial HsGrx2 is inhibited by a 2Fe–2S cluster that bridges two N-terminal active site Cys^{37} residues from separate molecules to form an inactive dimer. However, under oxidative stress, the iron–sulfur cluster dissociates readily to re-activate the enzyme [121,122]. The C-terminal Cys residue in the active site does not seem to be involved in iron–sulfur cluster assembly. In fact, Grxs that assemble iron–sulfur clusters with GSH are mostly the native mono-thiol forms that lack redox enzyme activity [37].

It is noteworthy that HsGrx1 has been shown to be a major player in copper metabolism in neuroblastoma cells [39]. In addition, HsGrx1 regulates the activity of the copper-transporting P-type ATPases ATP7A and ATP7B and protects neuronal cells from copper-induced toxicity [40–42]. Its Cu(I)-binding properties suggest that Grx1 may function as an alternative copper chaperone, linking copper homeostasis with redox homeostasis. Atox1 is a copper metallo-chaperone for the human copper ATPases ATP7A and ATP7B, but is not absolutely required for these ATPases to transport copper across the *trans*-Golgi network [123]. Could HsGrx1 be recruited as a back-up alternative copper chaperone? Certainly, HsGrx1/GSH have been shown to possess a functional partnership with the copper export machinery in human cells [58].

Concluding Remarks and Perspectives

Reversible thiol–disulfide exchanges act as redox switches for protein thiols and are involved in a wide spectrum of integrated cellular functions including redox sensing, cell signaling and anti-oxidative defense essential for redox homeostasis. Grxs have evolved as a class of versatile enzymes that catalyze these reactions utilizing GSH/GSSG as an electron donor/acceptor. The ubiquity and abundance of GSH in living cells emphasize the importance of such reactions in redox biology. The overall properties of Grxs allow them to catalyze both thiol–disulfide exchange reactions inherent in Fig. 1. The particular direction favored is determined by the properties of the protein substrate, the driving force defined by the GSSG/2GSH couple and also the medium conditions such as the presence of heavy metal ions in certain cases.

The versatility of Grxs in catalysis relies on several aspects including the following: (1) possession of intermediate reduction potentials that are GSH-dependent (especially for the catalytically competent disulfide form Grx(SH)(SSG)); this enables them to act as either a reductase for disulfide reduction or an oxidase for protein thiol oxidation; and (2) shuttling readily between the three oxidation states $Grx(SH)_2$, Grx(SH)(SSG) and Grx(SS) during catalysis; consequently, they are highly flexible in their catalytic function via either the monothiol or the dithiol mechanisms or both in parallel.

A systematic study and analysis suggested that favorable electrostatic and complementary surface interactions between the protein partners are the key determinants of substrate specificity [69]. This same principle may be applied to the examples given above on the catalytic reduction of RNR [69]. Indeed, the groups of oxidoreductase enzymes showing substrate specificity toward both PAPS reductase and RNR are strikingly similar [69]. A conclusion from these examples is that the C-terminal Cys in dithiol Grxs does not just simply act as a catalytic

brake: it plays an important role in catalysis when the monothiol catalytic route is blocked. However, for catalytic oxidation/reduction of a surface-exposed protein dithiol/disulfide such as those in HMA4n and Atox1 (Fig. 2), the monothiol mechanism is more efficient for both HsGrx1 and EcGrx1 [31,33].

Grxs may also function as Cu(I) binding proteins with the active site dithiol as the metal-binding motif. However, metal binding to the active site inhibits thiol–disulfide oxidoreductase activity. Consequently, Grxs may function either as redox enzymes controlling cellular redox homeostasis or as copper binding proteins assisting in maintaining copper homeostasis. On the other hand, Grxs that are important in Fe–S cluster assembly and in iron homeostasis and metabolism are generally the native monothiol forms that are in most cases catalytically inactive in classical Grx activity assay [37].

The relative concentrations of GSH and GSSG vary considerably across different cellular compartments and between intracellular and extracellular environments. Consequently, so do the formal reduction potentials imposed by the GSSG/2GSH redox couple [14,67,124]. However, cellular thiol–disulfide exchange processes usually work under kinetically controlled conditions that deviate from thermodynamic equilibria [66,67]. In this sense, Grx enzymes must play important roles in facilitating these essential dynamic cellular redox processes. In fact, the rapid response of Grxs to GSH levels under varying cellular conditions has been exploited to construct biosensors for real-time imaging of the GSH redox states in different cellular compartments [125].

However, many details regarding the mechanisms that drive the cellular functions of Grxs remain poorly understood. One of the main obstacles is a lack of reliable and convenient detection probes to follow disulfide bond exchange. The classic HEDS assay developed over two decade ago remains the most common assay for the Grx activity [23,112]. Development of new assays capable of monitoring disulfide bond exchange and mimicking Grx cellular functions remains a key challenge. Encouraging progress is being made [31–33,54,126].

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Abbreviations used:
DsbA, disulfide bond protein A; Grxs, glutaredoxins; GSH, glutathione; GSSG, glutathione disulfide; HEDS, bis(2-hydroxyethyl)disulfide; SOD, superoxide dismutase; $NADP^+$, nicotinamide adenine dinucleotide phosphate; NADPH, the reduced form of $NADP^+$; PDI, protein disulfide-isomerase; RNR, ribonucleotide reductase; ROS, reactive oxygen species; Trxs, thioredoxins; TrxR, thioredoxin reductase.

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